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Authors

Xiao, Yue-E
Jin, Dongmei
Jiang, Kai
et al.

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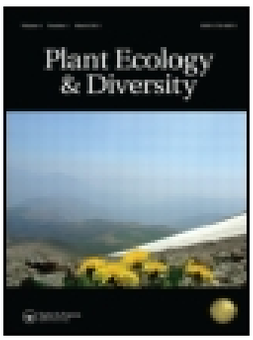

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ARTICLE

Pollinator limitation causes sexual reproductive failure in *ex situ* populations of self-compatible *Iris ensata*

Yue-E Xiao[a,b], Dongmei Jin[c], Kai Jiang[c], Yong-Hong Hu[c], Xin Tong[a], Susan J. Mazer[d] and Xiao-Yong Chen[a,e]

[a]Shanghai Key Laboratory for Urban Ecological Processes and Eco-Restoration, School of Ecological and Environmental Sciences, East China Normal University, Shanghai, China; [b]Shanghai Botanical Garden, Shanghai Engineering Research Center of Sustainable Plant Innovation, Shanghai, China; [c]Shanghai Chenshan Plant Science Research Center, Chinese Academy of Sciences, Shanghai Chenshan Botanical Garden, Shanghai, China; [d]Department of Ecology, Evolution and Marine Biology, University of California, Santa Barbara, CA, USA; [e]Shanghai Institute of Pollution Control and Ecological Security, Shanghai, China

ABSTRACT

Background: The absence of pollinators may prevent sexual reproduction and affect the distribution and persistence of individual plant populations, but the role of pollinators in shaping the patterns of distributions of plant species or populations has not been well studied.
Aims: Here, we tested the hypothesis that failure of sexual reproduction due to the absence of pollinators may contribute to the disjunct distribution of *Iris ensata*.
Methods: We assessed floral traits and pollinator visitation frequencies in three native and two *ex situ* populations. We also conducted controlled pollination experiments and examined progeny performance to estimate pollen limitation (PL) and inbreeding depression.
Results: Hand-pollinations indicated that *I. ensata* was a partially self-compatible outcrosser. Sexual reproduction in our study region depended on *Bombus trifasciatus*. We observed inbreeding depression for seed set and germination, but not for seedling survival. Native populations had higher frequencies of pollinator visitation and lower pollen-limitation of seed set than *ex situ* populations. Among populations, the magnitude of PL was negatively related to pollinator visitation rate ($P = 0.04$).
Conclusions: Pollinator limitation and inbreeding depression may both restrict the southern distribution of *I. ensata*, preventing its northward expansion, thus explaining the disjunct distribution of *I. ensata* in East Asia.

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Bumblebee; inbreeding depression; *Iris ensata*; long nectary; long-tongued pollinator limitation; restricted distribution

Introduction

Understanding species distributions and their underlying determinants is a central goal of ecological and biogeographic studies. Climate has been widely recognised as one of the most influential factors determining species distributions (Grinnell 1917), and thus the relationship between the presence of species and climatic variables has been used to predict species occurrence across space and/or time (Elith and Leathwick 2009; Shi et al. 2014). In addition to abiotic factors, biotic interactions can influence species distributions (e.g. Ettinger and Hille Ris Lambers 2013; Wisz et al. 2013; Hargreaves et al. 2014; Louthan et al. 2015). Theoretical and empirical studies have pointed out that strong antagonistic interactions among species such as competition, predation and parasitism (Algar et al. 2013) may exclude species from suitable habitats and thus limit their geographic range (Holt et al. 2011). However, some synergistic biotic interactions, such as facilitation and mutualism, may promote the survival of interacting species in otherwise unsuitable habitats and thus expand their realised niches (He et al. 2013; Afkhami et al. 2014; Tong et al. 2018). For example, at its high-latitude distribution limit, salt marsh vegetation may protect seedlings from fatally low temperatures, facilitating the range expansion of black mangrove (Guo et al. 2013).

Pollinator–plant interactions may influence the distribution of plant species for which seed production depends on animal pollen vectors and for which long-term population persistence depends on seed production (rather than clonal growth). For such species, the absence of pollinators will negatively affect population viability at sites that are otherwise suitable for survival and growth. The observation that Iridaceae species that have become naturalised exhibit higher autonomous fruit and seed production than congeneric, pollinator-dependent, non-naturalised species (van Kleunen et al. 2008) suggests that pollinators may be important in determining the colonisation ability of some Iridaceae species.

CONTACT Xiao-Yong Chen xychen@des.ecnu.edu.cn; sjmazer@ucsb.edu
Supplementary materials can be accessed here.

Understanding the factors that restrict the expansion of species beyond the boundaries of their current geographic ranges is critical, especially in the face of projected range shifts caused by climate change. If, for example, in response to a deteriorating climate, a species cannot successfully colonise new regions within its current range, then its long-term conservation will require intervention and active management. One factor that could limit the ability of a species to shift its geographic range would be the absence of effective pollinators within the region that otherwise the species could colonise. The role of pollinators in limiting species expansion has rarely been evaluated (e.g. Pauw and Bond 2011; Moeller et al. 2012). Pauw and Bond (2011) have demonstrated that pollination mutualism acted as a biotic filter that excluded non-clonal taxa from pollinator-limited communities. In the annual plant *Clarkia xantiana* A. Gray ssp. *xantiana* (Onagraceae), pollination limitation intensified as pollinators declined from the centre to the margin of its geographic range, indicating that pollinator-dependence may be an important constraint limiting range expansion in this subspecies (Moeller et al. 2012). It is also known that the magnitude of pollinator limitation may depend on the degree of plant-pollinator specialisation; taxa with more specialised pollination systems have been found to exhibit higher levels of pollen limitation (PL) due to the spatio-temporal variation in the abundance of pollinators (Knight et al. 2005).

Iris is the largest genus of the family Iridaceae, with nearly all its 260–300 species found in the temperate zone in the Northern Hemisphere. Most *Iris* species are self-incompatible (Avishai 1977; Avishai and Zohary 1980; Sapir et al. 2005; Pellegrino 2015), while some are self-compatible, with selfing rates ranging from 21.4% (Kron et al. 1993) to 71% (Planisek 1983). Most irises have the capacity for clonal growth, which may enhance rates of geitonogamous selfing in self-compatible species. In both self-compatible and self-incompatible irises, hand-pollination can increase seed set, indicating pollen-limited seed production (Wheelwright et al. 2006; Pellegrino 2015). Moreover, severe PL due to low pollinator availability may influence the mating system in *Iris*. For example, in the self-compatible *I. versicolor*, in island populations with low pollinator abundances, increased rates of autonomous selfing have been observed relative to mainland populations (Wheelwright et al. 2006).

Iris ensata Thunb. (Iridaceae), a perennial herb, is the ancestral species of numerous horticultural cultivars known as 'Japanese irises' or 'Hanashobu' (Hu and Xiao 2012). This species grows naturally in a cold and wet climate. It has a disjunct distribution in East Asia, where its primary range comprises eastern Russia, the Korean peninsula, Japan and north-eastern China (Zhao et al. 2000). Additionally, this species is also found on mountaintops of subtropical China, about 700 km from the edge of its northern distribution. The region separating the two ranges is climatically suitable for the survival of *I. ensata*, even though it is at much lower elevation (see Figure S1); for example, *I. ensata* grows well and flowers in Shanghai Chenshan Botanic Garden, about 180 km north of the edge of its current southern distribution. This cultivated population, however, produces no seeds. In this paper, we tested the hypothesis that sexual reproductive failure due to a lack of pollinators plays an important role in excluding *I. ensata* from the region between its northern and southern distributions.

Pollen-limited seed set may be mediated by either the receipt of pollen of low quality (e.g. self pollen that performs poorly), or by pollen deficiency due to the absence of, or low visitation by, pollinators (Aizen and Harder 2007). Pollinator scarcity is a common cause of reduced seed production in animal-pollinated plant populations, but it has seldom been reported that the total absence of pollinators can cause complete reproductive failure (Wilcock and Neiland 2002). To understand the potential mechanism of sexual reproduction failure, we performed controlled pollination experiments and identified the effective pollinator-(s) within the current geographic range of *I. ensata* and in adjacent areas outside of its range. Specifically, we asked the following questions: (1) Is there any difference in pollinator visitation between native and *ex situ I. ensata* populations? (2) Does low pollen deposition or low pollen quality prevent or reduce seed production in native or in *ex situ* populations? (3) If so, is seed set more limited in *ex situ* populations than in native populations? (4) Does self-pollination reduce seed set or offspring fitness, indicating that successful recruitment may be prevented by poor pollen quality? In addressing these questions, we also provide new information on floral phenotype and development in *I. ensata*, focusing on traits (e.g. herkogamy, dichogamy and nectary tube length) that may influence the mating system and degree of pollinator-dependence of *I. ensata*.

Materials and methods

Study species

Iris ensata is naturally found in wetlands. The inflorescences of *I. ensata* contain two symmetrical six-lobed flowers, which grow on a peduncle. Flowers are comprised of three pollination units, each of which consists of one inner petal, one stamen, one stigmatic lobe enclosed by one petaloid style branch and one outer petal (Figure S2). The pollination channel is composed of one style arm and one outer petal. The petals fuse at the base, forming the nectary, with the style located in the centre of the tube. The nectary sits upright on top of the ovary, and its walls produce nectar that accumulates at its base (Wesselingh and Arnold 2000). The nectary is connected to each pollination unit by two small openings, which are located on each side of the stamen filament (red arrow in Figure S2). The bright yellow signal guide is located at the base of the reddish-purple outer petal, guiding the pollinator to land and to enter the pollination tunnel.

Iris ensata flowers from mid-June to early July at the sites studied here (described below). In general, the inflorescence bears two flowers that collectively flower over 3 to 5 days. Each flower lasts for 2 or 3 days, depending on weather conditions. Fruit maturation and seed release occur from late September to early October.

Study sites

Based on extensive surveys, we located six natural populations in montane wetlands in the Tianmu Mts, Zhejiang Province, China (Figure S1). The Tianmu Mts are situated at 118° 36′–120° 06′ E, 29° 52′–30° 55′ N, with a maximum elevation of 1506 m above sea level (a.s.l.). Annual rainfall is 1300 and 1500 mm and mean annual temperature is 14.5 and 9.0°C at 350 m a.s.l. and 1506 m a.s.l., respectively (data from the Tianmu Weather Station, 1988–1996). We carried out our study in three native populations and two *ex situ* populations (Table S1). The distances between native populations (Dashigu (DSG), Taohuayuan (THY) and Tianchi (TC)) ranged 16.0–50.7 km, and the two *ex situ* populations (Pingyao (PY) and Chenshan (CS)) were ca. 49.1 km and 181.0 km, respectively, from population DSG. The two *ex situ* sites, i.e. CS (Shanghai) and PY (Hangzhou), are at lower elevations (ca. 20 m and 100 m a.s.l., respectively). The two *ex situ* populations (PY: 150 m^2, CS: 200 m^2) were established using seedlings germinated in the greenhouse from seeds collected from different patches in the DSG population in October of 2007. The density was 16 clones m^2 in the two *ex situ* populations, and flowers first appeared in 2009. Mean annual rainfall of Hangzhou and Shanghai is 1159.2 mm and 1378 mm, respectively. The mean annual temperature in Hangzhou and Shanghai is 17.8°C and 17.6°C, respectively (http://tianqi.eastday.com/news/37075.html). The environmental conditions of the two *ex situ* populations were similar to the habitats of natural *I. ensata* populations; the soils were rich in humus, slightly acidic and continuously moist in full sun (Hu and Xiao 2012). In addition, manual weeding was implemented in the *ex situ* populations.

Pollen and stigma viability

Traits measured in a single native population

Several floral traits were recorded from one of the sampled *ex situ* populations (CS) to characterise features that influence potential for autonomous self-pollination in *I. ensata* and hence its degree of reproductive assurance vs. pollinator dependence. First, we measured pollen and ovule production per flower to estimate the mean pollen to ovule ratio (P:O), which provides a proxy for mating system (Dafni 1992). Second, to measure the degree of dichogamy in this population, we examined the durations of pollen viability and stigma receptivity.

We estimated the P:O in 30 buds collected from different clones (Dafni 1992). Ovule number per flower was recorded using a microscope under 10× magnification. To estimate the number of pollen grains, anthers were sampled before anthesis and pollen grains of one intact anther per flower were gently transferred into 2 ml FAA solution with forceps and vibrated with an oscillator at 500 rpm (MS1Minishaker) for 1 min. 100 µl of the pollen grain solution was then placed in a haemocytometer and the number of pollen grains in solution was counted using a microscope with a magnification of 40×. Three 100 µl samples were counted from each 2 ml sample to obtain an estimate of the total number of pollen grains per anther. This number was then multiplied by three to estimate the total number of pollen grains per flower.

We estimated pollen viability using the TTC (2,3,5-triphenyl tetrazolium chloride) staining method (Dafni 1992). Under ambient conditions (25°C), 65 fresh anthers were collected from a total of 65 individuals in population CS between 8:00 and 9:00 am, as soon as the sepals opened, and were used to test pollen viability at each of 13 time periods after anthesis, i.e. 0,

1, 2, 4, 6, 8, 12, 24, 28, 32, 36, 48 and 72 h, respectively. Each time period was treated as an experimental treatment. Pollen grains from five anthers sampled from different clones (one anther per clone) located at least 5 m apart (5 clones × 13 time periods = 65 individuals) were mixed in each treatment (five replicates per treatment) and each sample was observed under a microscope at a magnification of 40×. Pollen viability was estimated as the percentage of all pollen grains that were viable (viable grains were identified based on their reddish stain).

Stigma receptivity was also determined by enzymatic activity identification (Dafni 1992). In the CS population, 10 stigmas from 10 different individuals freshly collected at 0, 1, 2, 4, 6, 8, 12, 24, 28, 32, 36, 48 and 72 h after flower opening, respectively, were dipped into an H_2O_2 solution (1% Benzidine: 3% H_2O_2:H_2O = 4:11:22), and stigmas were considered to be receptive if bubbles appeared (Dafni 1992). A total of 10 stigmas from 10 different individuals were measured at each time of stigma collection, and each stigma selection time was considered to be one treatment.

Traits measured in all study population

Several floral traits were recorded in all populations. A total of 10 to 15 fully open flowers were selected from randomly chosen plants, separated by at least 2 m (one flower per individual) in each population; these plants were sampled outside of the plots established to measure pollinator visitation (described below). Heights of one randomly selected stamen and stigma, nectary tube length and the maximum lengths and widths of the outer and inner petals of each flower were measured to the nearest 0.1 mm (Figure S2). We estimated the anther-stigma distance, which indicates the potential for reproductive assurance in each population. Nectary tube length was measured to determine whether the nectar could be reached by insects observed to visit the flowers. Given that the *ex situ* populations were established from seeds collected from one of the native populations (DSG), we did not expect to observe a difference in these floral traits between the *ex situ* populations and their seed source. We compared the floral biology of flowers sampled from each population, however, to confirm this expectation. Sampling information was shown in Table S2.

Flower density and visitation

At peak blooming in 2012, when about 70% flowers were open, we estimated floral density by setting up 10 (THY, TC) or 20 (DSG, PY and CS) plots per population, depending on population size. We randomly selected 1 m × 5 m plots in each population and recorded the number of open flowers displayed during the periods of pollinator observations. Visitation behaviour and the frequency of insect visitors in each population were observed during the blooming period of *I. ensata*, from 5 June to 1 July 2012 and from 9 June to 4 July 2013 (Table S3). Visitors that contacted both anthers and stigmas were considered to be legitimate pollinators. Three or five individuals of each visitor species were captured in each population and identified to the species level. We measured the tongue length of a total of 10 individuals of the presumed legitimate pollinators.

The floral visits were all recorded by video camera. Each camera was set on a tripod ca. 1.5 m away from each target plot to avoid shadows and the disturbance of insect visitors. In each population, two or three plots were randomly assigned for simultaneous observation. The cameras were moved to different plots every 2–3 h, and thus 6–20 plots were observed in each population. Observations were conducted on clear days from 08:00 to 16:00 h for two to four days at each population. We recorded 220.3 h in total. When the observations were finished, all videos were replayed in the laboratory. The arrival and departure times of each pollinator to each pollination tunnel were recorded to calculate the duration of each visitation. The visitation rate of each pollinator species was calculated as the mean number of visits to each flower per hour.

Breeding system and PL

Five pollination treatments were made in each population when *I. ensata* was in peak bloom in 2012 and 2013. *Iris ensata* can spread vegetatively by producing rhizomes that are usually less than 10 cm long between shoots. Thus, the shoots are generally clumped together, a phalanx-like structure here defined as a patch. A total of 30 to 50 patches were randomly chosen within the large (DSG, PY and CS) or small sites (THY and TC), and the distance between adjacent patches was ≥2 m. This distance is great enough to ensure that the patches represented different clones (Xiao 2014). Five flowers per patch were randomly tagged and each was assigned one of the following treatments: (1) open pollination or natural control, which had no manipulation; (2) spontaneous self-pollination, in which the flower was bagged before anthesis and had no other treatment; (3) apomixis, in

which the flower was emasculated and bagged before it opened; (4) self-pollination, in which the flower was hand-pollinated with geitonogamous pollen grains and bagged to exclude pollinators; (5) cross-pollination, in which the flower was bagged in advance on the day before it opened and hand-pollinated with pollen grains collected from three patches located 5 m away in the same population. To carry out this hand-pollination, three receptive stigma lobes per flower were each gently rubbed with a newly dehiscent anther. All bags (15 cm × 20 cm, mesh size 0.18 mm) were removed when flowers withered. All tagged fruit capsules were collected before they dehisced; capsules were collected in middle and in late September, in *ex situ* and native populations, respectively.

In each population, fruit set (the proportion of flowers that set a fruit with at least one seed), seed set and mean individual seed mass were quantified for each pollination treatment. If an empty fruit exhibited evidence of natural damage or fruit predators, it was discarded. Seed set of each treatment was expressed as the proportion of ovules per flower that developed into seeds (seed set = seed number/ovule number) (Becker et al. 2011). Flowers that did not develop into a fruit were treated as fruits with 0 seeds per flower.

Progeny performance

On 5 October 2012, seeds of each fruit were separately mixed with wet sand, placed in an open zip-top plastic bag and stratified at 4°C in darkness for 12 weeks. In early January 2013, seeds of each fruit were separately sowed in 15 cm × 15 cm pots filled with a mixture of coconut chaff, common gardening peat soil and perlite (4:1:5 by volume). The pots were placed at 20°C under a 12 h day/12 h night light regime in the Chenshan Botanical Garden greenhouse. Seeds started to germinate about one week after sowing; the number of emergent seedlings was recorded every three days. Survival rate and absolute growth rate based on fresh weight were estimated 100 days after sowing.

Data analyses

One-way analysis of variance (ANOVA) or Kruskal-Wallis *H* tests (for non-normally distributed data) were carried out to detect significant differences among populations with respect to each morphological trait. The difference between stigma and anther heights for each flower was tested using a linear mixed model (LMM), considering population as a random effect.

We used LMMs to detect the factors that significantly affected visitation rate and duration. Fixed factors were population type (natural and *ex situ*) and year, while population was treated as a random factor. We used generalized linear mixed models (GLMMs) to test the factors that significantly affect fruit set, seed set, mean individual seed mass, germination, survival and growth rate. Fixed factors were population type (natural and *ex situ*) and treatment (control, selfing and outcrossing), and patch nested within population was treated as the random factor. The model also included the effect of patch (nested within populations) and the pollination treatment × population type interaction. Furthermore, in each population, GLMMs were conducted to detect variation in seed number per flower, seed set per flower, mean individual seed mass and progeny performance (germination rate, survival rate and growth rate) due to pollination treatments (control vs. crossing-pollination and selfing- vs. crossing-pollination). The distribution of residuals was assumed to be binomial for fruit set, seed set, germination and survival; Poisson distribution for seed number per flower, and to be Gaussian for seed mass and growth rate.

Self-compatibility index (SCI) was calculated following Lloyd and Schoen (1992): SCI = fruit set of self-pollinated flowers/fruit set of cross-pollinated flowers. PL is the proportional reduction in seed set observed in open-pollinated flowers relative to hand-pollinated, outcrossed fruits, and was calculated for each patch as follows: PL = 1–(So/Sc), where So and Sc represent seed set (the proportion of ovules that develop into seeds) following open- and cross-pollination, respectively (Larson and Barrett 2000). PL at the population level was calculated as the mean PL among all patches. One-way ANOVA was used to detect a significant difference between native and *ex situ* population with respect to mean PL.

We calculated inbreeding depression (δ) to evaluate the fitness reduction of selfed (w_s) relative to outcrossed offspring (w_o): $\delta = 1-(w_s/w_o)$ (Ågren and Schemske 1993). δ was calculated for seed set (δ_{ss}), seed germination (δ_{sg}), seedling survival (δ_{su}) and growth (δ_{gr}). Cumulative inbreeding depression (δ_{cu}) was similarly calculated (Ågren and Schemske 1993). Tukey's multiple comparisons were used to detect significant differences among populations in mean visitation frequency, PL and

inbreeding depression. Spearman's correlations test was used to examine the correlation among populations between PL and pollinator visitation rate.

All analyses were made using the statistical environment *R* (www.r-project.org).

Results

Pollen and stigma viability

Iris ensata flowers exhibit intermediate levels of protandry. The floral lifespan was 52.4 ± 0.5 h (range from 46 to 60 h; n = 50). As the three outer petals reflexed, the anthers dehisced and released pollen with a mean viability of 96.1 ± 1.7% (n = 5) (Table S4). Pollen viability decreased to 54.7 ± 2.5% and 28.0 ± 3.6% (n = 5) at 24 and 48 h post-blooming (hpb), respectively. The stigmas became receptive ca. 6–8 hpb. Stigma receptivity was highest at 24–28 hpb (Table S4), and the stigmas began to wither and become enveloped by a wilting petal at ca. 36 hpb. Thus, within individual flowers, the male and female stages overlapped from 6–36 hpb, while the male stage functioned alone from 0–6 and 36–48 hpb. Mean (±SE) ovule number per flower was 98.99 ± 0.65 (n = 373). The mean pollen-ovule ratio was 1426.9 ± 71.4 (n = 30, range: 1015.6–2500.0), and Dafni's (1992) outcrossing index was 4, suggesting that *I. ensata* is facultatively xenogamous.

Floral traits and pollination system

In the two *ex situ* populations (PY, CS), which are located at relatively low elevations, *I. ensata* flowered and fruited two weeks earlier than in native populations, which are located at sites >950 m a.s.l. (Table S1). Among all *I. ensata* populations studied, the width and length of the outer petals were 36.9 ± 0.4 and 90.1 ± 0.5 mm, respectively, and the inner petals were 4.1 ± 0.5 mm in width and 45.1 ± 0.7 mm in length (n = 120). There were no significant differences among populations in mean petal width (outer petal: $F_{4,115}$ = 0.691, P = 0.65; inner petal: $F_{4,115}$ = 1.827, P = 0.13) or length (outer petal: $F_{4,115}$ = 0.672, P = 0.61; inner petal: $F_{4,115}$ = 0.511; P = 0.77). Mean nectar tube length was 13.5 ± 1.0 mm (range: 11–15.3 mm), and did not differ significantly among populations ($F_{4,115}$ = 1.602, P = 0.18). Stamens were lower than the paired stigmatic lobes, and the stamen-stigma distance was 8.3 ± 0.3 mm (mean ± SE), indicating strong herkogamy. There was no significant difference between native and *ex situ* populations in the stamen-stigma distance ($F_{4,115}$ = 1.973, P = 0.10).

Flower density and visitation

Bombus trifasciatus was the only species observed that contacted both anthers and stigmas during the experiment and is therefore considered to be the only legitimate pollinator in the studied populations. This species appears to be guided by the bright yellow signal to land on a petal; the bee then opens the petaloid style with its head and enters the pollination tunnel along the yellow guide (Figure S3a). Its tongue is long enough (11.0 ± 1.3 mm) to reach nectar when its head is near the base of the tunnel.

The mean number of flowers per plot was 9 and 22 among plots in natural and *ex situ* populations, respectively. The mean duration per visit by *B. trifasciatus* was 12.1 ± 2.3 s, which did not differ between population types ($F_{1,2.6}$ = 2.17, P = 0.25) or years ($F_{1,73.1}$ = 2.18, P = 0.14). However, pollinator visitation frequency was significantly higher in native than in *ex situ* populations ($F_{1,2.2}$ = 125.09, P = 0.005) (Figure 1). There was a significant difference in the mean visitation frequency of pollinators among populations ($F_{4,111.3}$ = 34.73, P < 0.001). The mean visitation rates of *B. trifasciatus* in native populations were 2.21 and 2.23 times·flower^{-1}·h^{-1} in 2012 and 2013, respectively; this was much higher than those of the *ex situ* populations. We observed no *B. trifasciatus* in one *ex situ* population (CS) and the mean visitation rates of *B. trifasciatus* in the PY population were 0.05 and 0.06 times·flower^{-1}·h^{-1} in 2012 and 2013, respectively.

Another bumblebee, the short-tongued *B. flavescens*, was also found, though rarely, in the native populations. This bumblebee occasionally alighted on the outer petals, but it never entered the pollination tunnel. An alien honeybee (*Apis mellifera*) was abundant in the two *ex situ* populations. However, we never observed honeybees enter the pollination tunnels from the entrance. Instead, honeybees would touch a stamen by entering the side of a tunnel that opened after the stigmas wilted and then feed on pollen grains (Figure S3b). During this process, the honeybees did not touch the (unreceptive) stigmas; thus, *A. mellifera* is a pollen robber of *I. ensata* and not a pollinator of it.

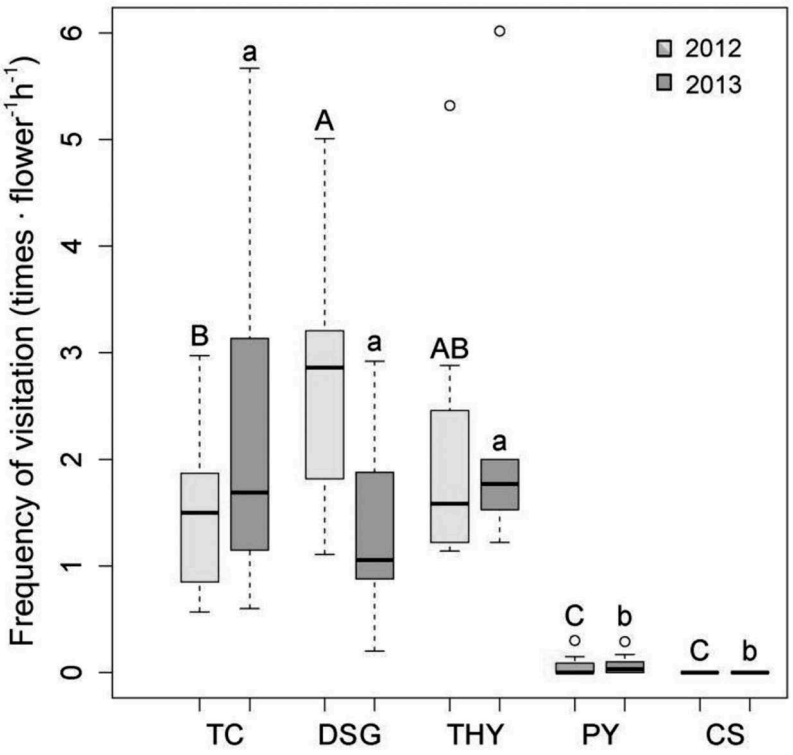

Figure 1. The visitation frequencies of pollinators (times flower^{-1} h^{-1}) in the five focal *Iris ensata* populations in 2012 (orange columns) and 2013 (grey columns). PY and CS are the *ex situ* populations. For each box-and-whisker plot, the box shows 25% median and 75% quantile of the given values. The whiskers extend to the most extreme data points that are not more than 1.5 times the interquartile range (length of the box) from the box. Upper case letters indicate pairwise comparisons among population means for 2012; lower case letters indicate pairwise comparisons among population means observed in 2013. Populations labelled with different letters differ significantly with respect to the mean frequency of pollinator visitation during the observed periods ($P < 0.05$).

Breeding system

Flowers hand-pollinated with pollen from geitonogamous pollinations set seeds, indicating that *I. ensata* is self-compatible. Bagged flowers, however, produced no seeds in any population, indicating that *I. ensata* exhibits no agamospermy or spontaneous autogamy.

Among the native populations, mean (±SE) fruit set of the open-, self- and cross-pollination treatments was 71.3 ± 5.4%, 67.2 ± 3.6% and 74.3 ± 5.1% ($n = 3$), respectively, and the SCI was 58.1%. By contrast, mean fruit set differed greatly among the pollination treatments in the two *ex situ* populations. Open-pollinated flowers had the lowest fruit set in these two populations. In population CS, no fruit was set in the open-pollination treatment, and fruit set of the self- and cross-pollination treatments was 61.7% and 72.3%, respectively. In population PY, fruit set was 24.5% for open-pollinated flowers, lower than for self- (67.3%) and cross-pollinated flowers (69.4%). Mean fruit set of natural populations was significantly higher than that of *ex situ* populations ($z = 4.507$, $P < 0.001$). However, no significant difference in fruit set was observed among cross-, self- and open-pollination among native populations.

In the three native populations (data pooled), mean seed number per flower (including aborted fruits) was 44.6 ± 1.7 ($n = 81$), 33.0 ± 1.5 ($n = 81$) and 56.9 ± 1.5 ($n = 81$) for open-, self- and cross-pollination, respectively (Table 1). In contrast, mean seed number per flower was 4.9 ± 1.4 ($n = 68$), 24.9 ± 1.4 ($n = 68$) and 45.0 ± 1.8 ($n = 68$) for open-, self- and cross-pollination, respectively, in the two *ex situ* populations. In general, there were significant difference between population types ($df = 1$, $\chi^2 = 32.95$, $P < 0.001$) and treatments ($df = 2$, $\chi^2 = 1089.3$, $P < 0.001$). The interaction between population types and treatments was also significant ($df = 2$, $\chi^2 = 1063.7$, $P < 0.001$). Open- and self-pollination led to significantly lower seed set than cross-pollination (open- vs. cross-pollination: $z = -38.54$, $P < 0.001$; self-pollination vs. cross-pollination: $z = -19.63$, $P < 0.001$).

In each population, seed set exhibited a pattern similar to that of seed number per flower (Table 1). Across all native populations,

Table 1. Mean values (±SE) of the number of seeds per flower, seed set per flower, mean individual seed mass, germination percentage, seedling survival and growth rate observed in each pollination treatment and population. The GLMM method was used to test for a significant difference between the control and hand cross-pollination treatments or between self- and cross-pollination treatments. The distribution of residuals was assumed to be binomial for data of fruit set, seed set, germination and survival, to be Poisson for seed number per flower, and to be Gaussian for seed mass and growth rate.

					P-value	
	Population	Open	Selfing	Crossing	Control vs. crossing	Selfing vs. crossing
(1) Number of seeds per flower (Mean ± SE)	TC	37.6 ± 3.6 (n = 20)	29.5 ± 3.9 (n = 20)	56.5 ± 3.8 (n = 20)	<0.001	<0.001
	DSG	47.8 ± 2.1 (n = 37)	34.4 ± 2.2 (n = 37)	56.7 ± 2.4 (n = 37)	<0.001	<0.001
	THY	45.7 ± 2.8 (n = 24)	33.6 ± 2.2 (n = 24)	57.2 ± 1.9 (n = 24)	<0.001	<0.001
	PY	9.8 ± 2.5 (n = 34)	27.0 ± 1.5 (n = 34)	46.5 ± 2.6 (n = 34)	<0.001	<0.001
	CS	0.0 ± 0.0 (n = 34)	22.7 ± 2.2 (n = 34)	43.5 ± 2.5 (n = 34)	<0.001	<0.001
(2) Seed set per flower (Mean ± SE)	TC	38.2 ± 4.0 (n = 20)	30.1 ± 4.1 (n = 20)	55.5 ± 3.9 (n = 20)	<0.001	<0.001
	DSG	48.6 ± 2.1 (n = 37)	34.5 ± 2.2 (n = 37)	56.1 ± 2.1 (n = 37)	<0.001	<0.001
	THY	46.0 ± 2.7 (n = 24)	36.3 ± 2.4 (n = 24)	56.9 ± 2.1 (n = 24)	<0.001	<0.001
	PY	9.5 ± 2.4 (n = 34)	28.6 ± 1.8 (n = 34)	47.8 ± 2.9 (n = 34)	<0.001	<0.001
	CS	0.0 ± 0.0 (n = 34)	23.5 ± 2.4 (n = 34)	46.1 ± 2.8 (n = 34)	<0.001	<0.001
(3) Seed mass (mg) (Mean ± SE)	TC	17.6 ± 1.4 (n = 18)	14.9 ± 1.7 (n = 16)	19.1 ± 1.4 (n = 20)	0.406	0.050
	DSG	18.2 ± 0.9 (n = 36)	17.9 ± 1.2 (n = 34)	20.7 ± 1.5 (n = 37)	0.120	0.102
	THY	18.0 ± 1.0 (n = 23)	17.3 ± 1.0 (n = 23)	19.5 ± 1.0 (n = 24)	0.097	0.054
	PY	16.3 ± 1.3 (n = 12)	13.8 ± 0.6 (n = 33)	15.6 ± 0.8 (n = 34)	0.318	0.047
	CS	–	14.0 ± 0.8 (n = 29)	14.9 ± 1.0 (n = 34)	–	0.320
(4) Germination percentage (Mean ± SE)	TC	54.0 ± 2.7 (n = 18)	46.0 ± 2.3 (n = 16)	54.8 ± 3.1 (n = 20)	0.483	<0.001
	DSG	57.2 ± 2.9 (n = 36)	50.5 ± 2.2 (n = 34)	56.0 ± 2.7 (n = 37)	0.791	0.003
	THY	61.9 ± 3.6 (n = 23)	52.6 ± 2.4 (n = 23)	64.1 ± 2.5 (n = 24)	0.074	<0.001
	PY	55.8 ± 3.1 (n = 12)	47.8 ± 2.5 (n = 33)	60.9 ± 2.4 (n = 34)	0.510	<0.001
	CS	–	43.0 ± 2.2 (n = 29)	52.6 ± 2.7 (n = 34)	–	<0.001
(5) Seedling survival (Mean ± SE)	TC	0.923 ± 0.024 (n = 18)	0.906 ± 0.019 (n = 16)	0.906 ± 0.016 (n = 20)	0.892	0.814
	DSG	0.917 ± 0.019 (n = 36)	0.928 ± 0.014 (n = 34)	0.931 ± 0.011 (n = 37)	0.054	0.968
	THY	0.881 ± 0.032 (n = 23)	0.892 ± 0.028 (n = 23)	0.933 ± 0.012 (n = 24)	<0.001	<0.001
	PY	0.834 ± 0.060 (n = 12)	0.811 ± 0.042 (n = 33)	0.895 ± 0.020 (n = 34)	0.010	<0.001
	CS	–	0.920 ± 0.031 (n = 29)	0.888 ± 0.025 (n = 34)	–	0.042
(6) Growth rate (mg/d) (Mean ± SE)	TC	0.677 ± 0.063 (n = 18)	0.692 ± 0.057 (n = 16)	0.654 ± 0.058 (n = 20)	0.906	0.125
	DSG	0.659 ± 0.48 (n = 36)	0.633 ± 0.049 (n = 34)	0.681 ± 0.050 (n = 37)	0.236	0.063
	THY	0.614 ± 0.037 (n = 23)	0.617 ± 0.053 (n = 23)	0.645 ± 0.053 (n = 24)	0.316	0.128
	PY	0.542 ± 0.067 (n = 12)	0.511 ± 0.038 (n = 33)	0.550 ± 0.037 (n = 34)	0.824	0.083
	CS	–	0.532 ± 0.030 (n = 29)	0.591 ± 0.037 (n = 34)	–	0.033

n refers to the number of flowers.

mean seed set per flower was 45.3 ± 1.6% (n = 81), 33.9 ± 1.6% (n = 81) and 56.2 ± 1.5% (n = 81) for open-, self- and cross-pollination treatments, respectively. In the *ex situ* populations, seed set was very low among open-pollinated flowers (4.8 ± 1.3%, n = 68), but higher for self- (26.1 ± 1.5%, n = 68) and cross-pollination treatments (47.0 ± 2.0%, n = 68). In the *ex situ* populations, seed set per flower in two pairs of orthogonal contrasts differed significantly between treatments (GLMM): (i) open- vs. cross-pollination (z = −13.51, P < 0.001) and (ii) self- vs. cross-pollination (z = −21.98, P < 0.001) (Table 1). Mean seed set of native populations was higher than that of *ex situ* populations (z = 4.413, P < 0.001) (Table 1). For seed set, the pollination treatment × population type interaction is highly significant (df = 2, χ^2 = 66.464, P < 0.001).

In contrast, mean individual seed mass did not differ between open- and cross- pollination treatments in each population ($F_{1,107.4}$ = 3.02, P = 0.085), but outcrossed flowers had higher mean individual seed mass than selfed flowers ($F_{1,142.09}$ = 13.78, P < 0.001) (Table 1). The mean individual seed mass of native populations was significantly higher than that of *ex situ* populations ($F_{1,2.6}$ = 25.20, P = 0.020) (Table 1), and the pollination treatment × population type interaction was not significant (GLMM, $F_{1,251.1}$ = 1.720, P = 0.181).

Self-pollination was associated with relatively low seed set and percent germination (Table 1). The mean germination percentage was 57.1 ± 1.7% (n = 89), 48.1 ± 1.1% (n = 149) and 57.6 ± 1.2% (n = 135), respectively, for open-, self- and cross-pollination treatments across all populations. In each population, the germination percentage of seeds produced by self-pollination was significantly lower than for seeds produced by cross-pollination (z = −7.61, P < 0.001). However, there was no significant difference in seed germination rate between open- and cross-pollination (z = −0.67, P = 0.51). Furthermore, native and *ex situ* populations did not differ significantly with respect to the percent germination of seeds produced by open- or cross-pollination (df = 1, χ^2 = 0.94, P = 0.33). Meanwhile, there was no significant pollination treatment × population type interaction (df = 2, χ^2 = 3.18, P = 0.16).

The fruit development rate following cross-pollination treatment was significantly higher than that of open-pollinated flowers (z = 3.133, P = 0.002), but did not differ significantly from fruit development rate following self-pollination (z = 1.578, P = 0.12). The survival rate of seedlings was significantly higher in native populations than in *ex situ* populations (z = 2.388, P = 0.016). No significant difference in seedling growth rate was detected between seeds produced by open- and self-pollinations ($F_{1,92.7}$ = 1.79, P = 0.18) or between population types ($F_{1,2.5}$ = 6.90, P = 0.09), but seedling growth rate was significantly higher among seeds produced by cross-pollination than by self-pollination ($F_{1,136.0}$ = 16.99; P < 0.001) (Table 1). There was no significant pollination treatment × population type interaction on seedling growth rate ($F_{2,227.1}$ = 0.39, P = 0.68).

Pollen limitation

Open-pollinated flowers had lower seed production than hand-pollinated, outcrossed flowers in all populations (Table 1). PL (estimated here as the reduction in seed production in open-pollinated relative to hand-pollinated, outcrossed flowers) in *ex situ* populations was significantly higher than in native populations (based on one-way ANOVA; $F_{4,143}$ = 382.8, P < 0.001) (Figure 2). PL was lowest in population DSG (PL = 0.13), which was significantly lower than that of all other populations except for THY. Population CS had the highest PL (PL = 1.0) since there was no fruit set of open-pollinated flowers. The other *ex situ* population (PY) ranked second in the magnitude of PL (PL = 0.81).

Populations with higher pollinator visitation rates exhibited less pollen-limitation of seed set (r = −0.857, P = 0.04). There was no correlation among populations, however, between flower density and PL of seed set (P = 0.39).

Inbreeding depression

Among all five populations, the mean inbreeding depression coefficient (δ) at the seed set stage was 0.41. Mean δ for percentage seed germination was 0.16. The mean cumulative inbreeding depression of seed set and germination was 0.50.

At the population level, mean seed set and germination percentage of outcrossed progenies were significantly higher than those of selfed progenies, but there was no significant difference in seedling survival and growth between self- and cross-pollination treatments (Table 1).

Inbreeding depression for seed set did not differ significantly among populations (Figure 3). However, for germination rate, inbreeding

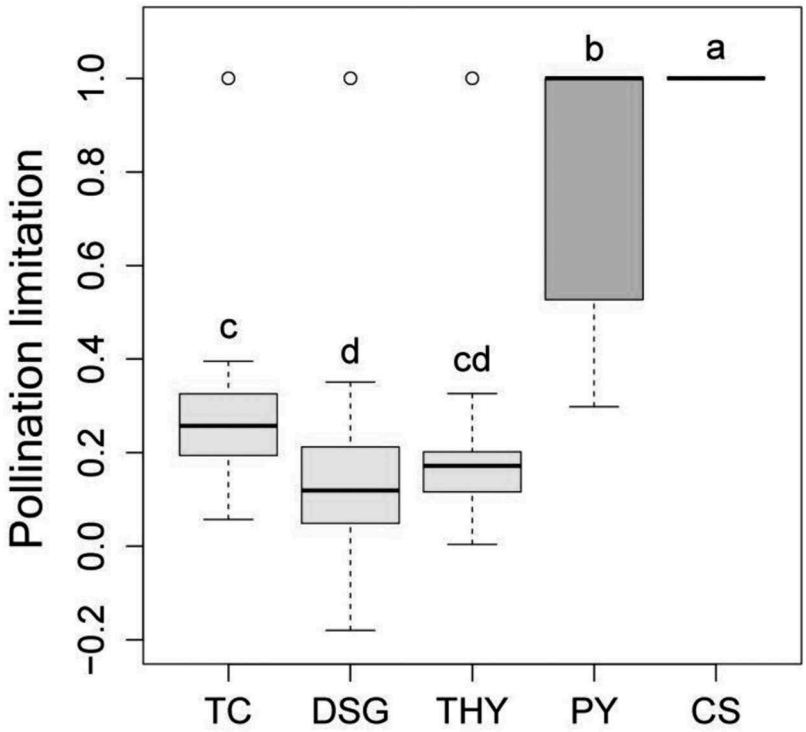

Figure 2. Difference in seed set between open-pollinated and hand-pollinated, outcrossed flowers in each population of *Iris ensata*, estimated as the mean seed set observed in the hand-pollinated treatment minus the mean seed set observed in the open-pollinated treatment. We interpret this difference as the magnitude of pollen limitation (PL) of seed set, but we cannot determine whether the lower seed set of open-pollinated flowers is due to lower pollen quantity or lower pollen quality. Native and *ex situ* populations are in grey and dark grey, respectively. Description of box-and-whisker plot is the same as Figure 1.

depression in the native population DSG was significantly lower than that of two *ex situ* populations ($P < 0.01$). Cumulative inbreeding depression in DSG was significantly lower than that of three of the other populations (PY, CS and TC) ($P < 0.001$).

Discussion

Based on the observations presented here, we propose that pollinator limitation leads to PL and inbreeding depression, both of which affect the geographic distribution of *Iris ensata*. The absence (in population CS) and very low abundance (in PY) of pollinators of *I. ensata* in the region occupied by the *ex situ* populations studied here might be due to its low altitude or to other abiotic or biotic factors, which may in turn prevent successful reproduction in *ex situ* populations of *I. ensata*.

Breeding system in Iris ensata

The moderate seed set observed following self-pollination indicates that *I. ensata* is partly self-compatible with a SCI of 58.1%. However, selfing is prevented in the absence of pollinators by high herkogamy. The pollen to ovule ratio is consistent with facultative xenogamy (Dafni 1992), as confirmed by the observation that autonomous selfing is prevented by herkogamy, whether or not flowers are emasculated. The failure of *I. ensata* to produce seeds in the cultivated population at Chenshan Botanical Garden in Shanghai, where there are no effective pollinators, also supports the inference that spontaneous selfing does not occur in *I. ensata*. However, pollen vector-mediated selfing is possible. Under natural conditions, selfing in *I. ensata* may occur by way of two processes: autogamous and geitonogamous selfing. The former may occur as a pollinator withdraws from a flower, if pollen adhering to its body is released onto the stigma. The latter may occur when a stigma is pollinated by insect-borne pollen grains transferred from other flowers on the same inflorescence or from other clonally produced ramets; such geitonogamous selfing is common in plants displaying extensive vegetative reproduction (e.g. Reusch 2001).

Iris ensata is effectively pollinated by only one species, *Bombus trifasciatus*, in the populations studied here. The tightly closed pollination channel, formed by a petaloid stigma branch and its corresponding outer petal, may block small pollinators from entering. *Bombus trifasciatus* is strong enough to open the entrance, to enter the channel

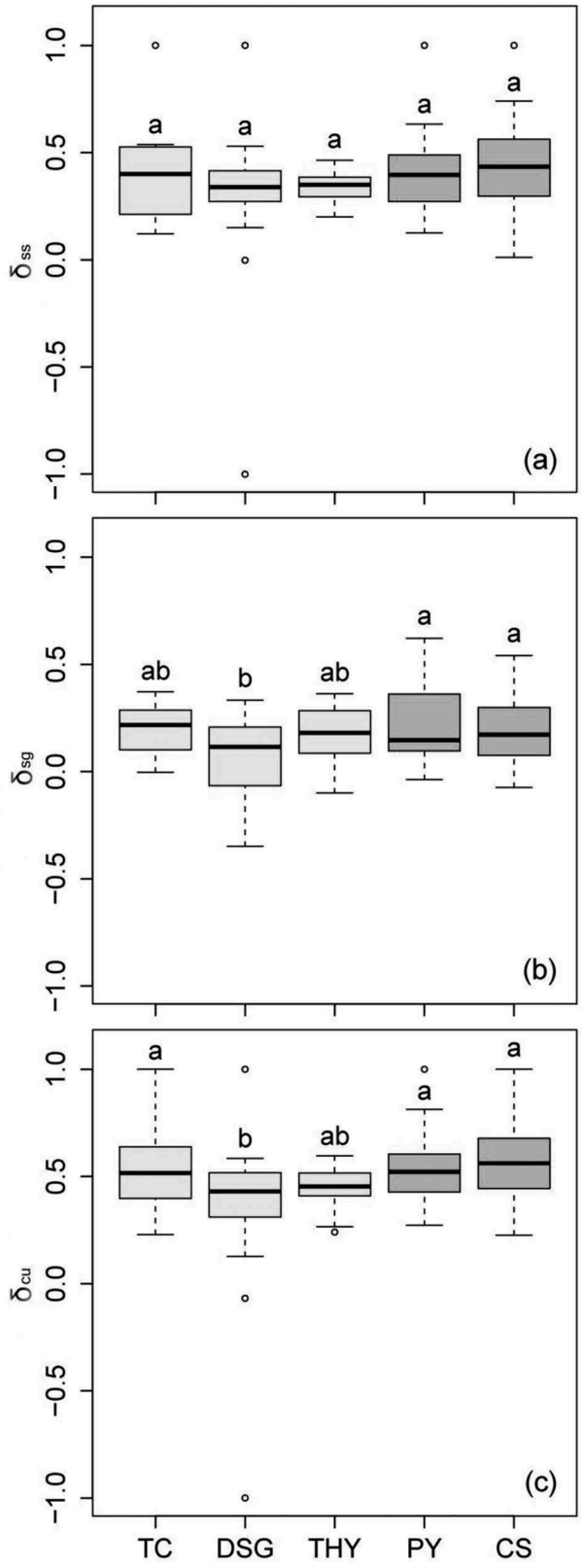

Figure 3. Inbreeding depression coefficients for two life stages (seed set (δ_{ss}) and seed germination (δ_{sg})) and cumulative inbreeding depression across these two life stages (δ_{cu}) in each *Iris ensata* population. Native and *ex situ* populations are in grey and dark grey, respectively. Description of box-and-whisker plot is the same as Figure 1.

to feed on the nectar and to pollinate the flowers. The nectary tube of *I. ensata* ranges 11.0–15.3 mm long, and the tongue length of *B. trifasciatus* ranges 9.5–13.9 mm, indicating that its tongue is long enough to access nectar in *I. ensata*. In the region studied here, another common bumblebee, *B. flavescens*, is short-tongued (Williams and Jackson 2007) and cannot reach the nectar at the base of the nectary tube of *I. ensata*.

PL and inbreeding depression in natural populations

PL of fruit and seed set is common in wild populations (Ashman et al. 2004). Estimating the magnitude of PL from the difference in seed set or seed number between open-pollinated and hand-pollinated, outcrossed flowers, however, is problematic because the difference observed between treatments could be due to differences between them in pollen quantity and/or quality (Aizen and Harder 2007). In the current study, we did not record the number of pollen grains deposited on the receptive stigmas of the pollination treatments, so we cannot assert that they differed in the number of pollen grains received. In addition, we do not know what proportion of the pollen deposited on open-pollinated stigmas was self- vs. outcross pollen. As a result, we cannot distinguish between pollen quality and pollen quantity as alternative causes of the difference in seed production between the open-pollinated and the hand-pollinated, outcrossed treatments. Here, we interpret cases in which the mean seed set of hand-pollinated, outcrossed flowers exceeded that of the open-pollinated flowers as PL (Figure 3), but we do not know whether the lower seed set of the open-pollinated flowers was due to lower pollen quantity, pollen quality or both. Future studies should be designed to distinguish between these alternatives by quantifying pollen deposition, by estimating selfing rates in both open- and hand-pollinated (outcrossed) treatments, and by providing a true 'pollen supplementation' treatment in which open-pollinated stigmas receive additional pollen via hand-pollinations.

Another difficulty with interpreting the difference between open-pollinated and hand-pollinated, outcrossed flowers is that we did not control for the possibility that, in *ex situ* populations, the reallocation of resources from open-pollinated flowers receiving low levels of pollen to hand-pollinated, outcrossed flowers may have contributed to the large differences between pollination treatments in seed production, seed set and fruit set. Such reallocation can lead to over-estimates of the degree of PL when hand-pollinated treatments produce more seeds than they would have in the absence of

such re-allocation. In this study, however, the mean seed production of hand-pollinated, outcrossed flowers was similar in both native and *ex situ* populations even though the mean seed set of the open pollinated flowers in native populations greatly exceeded that in *ex situ* populations (Table 1). If the re-allocation of resources among flowers were common in the *ex situ* populations, one would have expected the hand-pollinated, outcrossed flowers in these populations to exhibit higher seed production than their counterparts in the native populations. This pattern, however, was not seen (Table 1). Nevertheless, although we demonstrated that, in all our sampled populations (and particularly in the *ex situ* populations), pollen quality or quantity limited seed set and seed production of individual flowers, we cannot conclude that these factors limit seed production at the level of individual plants. Consequently, we cannot assert that individual fitness in *ex situ* populations is necessarily limited by the quantity or quality of the pollen received.

The natural populations of *I. ensata* studied here exhibit low to moderate levels of PL for seed set, ranging 0.13–0.32 (Figure 2). This value is similar to the mean value of PL for fruit set observed in *I. tuberosa*, which ranged 0.18–0.26 among populations (Pellegrino 2015). Although estimates of PL based on seed set vs. fruit set are not equivalent, both types of PL may be due to either insufficient pollination or the receipt of low-quality pollen (Aizen and Harder 2007; Vamosi et al. 2013).

Inbreeding depression for seed production in natural populations of *I. ensata* (0.4) was similar to that observed in many outcrossing species (Husband and Schemske 1996). As mentioned above, *I. ensata* is self-compatible and facultative selfing is possible, particularly where clonal growth is extensive. In natural populations of *I. ensata*, the diameter of a genet is generally less than 5 m (Xiao et al. 2015), and seeds are usually dispersed only a short distance (ca. 0.5 m). As a result, the neighbours of an *I. ensata* individual are most likely to be close relatives or ramets of the same genotype. Thus, stigmas of *I. ensata* may receive abundant pollen grains from the same or closely related genotypes, resulting in lower than maximum seed set due to inbreeding depression.

Inbreeding depression in *I. ensata* was observed with respect to seed set and germination rate but not in seedling growth or survival. This result is consistent with other reports that inbreeding depression may differ among life history stages, and that its magnitude in outcrossing species is often highest during seed development (Husband and Schemske 1996). The level of inbreeding depression is related to self-compatibility in *Iris*. Wheelwright et al. (2006) have observed no inbreeding depression in percent fruit set, fruit size or the number of seeds per fruit in the self-compatible *I. versicolor*, while, in the self-incompatible *I. tuberosa*, fruit set following hand-pollination with self pollen was 10.8–15.2% of that following hand-pollination with outcross pollen (Pellegrino 2015). The absence of inbreeding depression in *I. versicolor* can be explained by the fact that deleterious alleles are more easily purged in taxa that are capable of selfing and inbreeding (therefore exposing such alleles in their homozygous form) than in taxa that are incapable of doing so. However, this may not hold in all taxa. For example, high lifetime inbreeding depression and a mixed mating system were observed in the self-compatible, long-lived perennial plant *Rhododendron ferrugineum* (Delmas et al. 2014).

Pollinator limitation in ex situ *populations: implications for the range limits of* I. ensata

Abiotic factors, such as climate and soil conditions, generally strongly affect plant growth and reproduction. The observation that *I. ensata* grows well, flowers normally and produces many seeds following hand-pollination with outcross pollen at two sites where it is cultivated indicates that they are climatically suitable for this species. In addition, *I. ensata* has a narrower realised geographic range than that predicted by environmental factors alone (Xiao 2014). *Bombus trifasciatus* is not found (or is very uncommon) in the region between the northern and southern ranges of *I. ensata*, most likely due to unfavourable climatic conditions. The temperature suitable for bumblebees ranges 7–25°C, and a number of *Bombus* species decline sharply at elevations below 500 m and above 4000 m in subtropical regions (Dao 2004). Consequently, we infer that abiotic factors are unlikely to account for the sterility of *I. ensata* in the *ex situ* populations examined here and that a lack of pollinators is a causal factor limiting the geographic range of this species. Other possible biotic reasons for the disjunct distribution of *I. ensata* include limited seed dispersal; competition with other plant species; poor soil quality in unoccupied regions (e.g. *I. ensata* may require mycorrhizae that are not widespread); and other biotic factors not examined

here. These factors may also explain the disjunct distributions of other species, such as *Gagea triflora* (Ledeb.) Roem. et Schult. and *Hylomecon japonica* (Thunb.) Prantl (http://frps.iplant.cn/).

In the two introduced populations of *I. ensata* adjacent to its native range, we observed strong PL (Figure 2). The negative relationship observed between PL and pollinator frequency across the populations evaluated here suggests that PL due to low pollen deposition or low pollen quality occurred in these *ex situ* populations. The PY population, about 50 km from the closest natural populations, had a very low pollinator visitation rate due to reduced pollinator density and extremely low seed set among open-pollinated flowers. In the CS population, no bumblebees were observed, and all open-pollinated individuals failed to produce seed. However, hand-pollination with outcross pollen achieved ca. 50% seed set, comparable to that in natural populations (Table 1).

These findings indicate that the absence or scarcity of effective pollinators prevented seed production in these *ex situ* sites. This conclusion is straightforward in species for which seed reproduction is pollinator-dependent, and plant species that rely on single, specialist pollinators may be more vulnerable to pollinator limitation than species visited by multiple species of pollen vectors (Knight et al. 2005). Pollinator limitation is common in *Iris*, even in self-compatible species or those pollinated by many insect species (Kron et al. 1993; Wheelwright et al. 2006).

Iris ensata is a species capable of both sexual and asexual reproduction. *Iris ensata* may expand via clonal growth, and persist for some time; however, without seedling recruitment, genetic diversity may decline due to natural selection (Eriksson 1993). Populations of low genetic variation have a high risk of local extinction, especially in a changing environment. Thus, without sexual reproduction, *I. ensata* may not persist in the long-term. During our study period, we found no obvious impacts of herbivory or diseases on growth and reproduction of *I. ensata*.

Implications

Given that the absence of a pollinator may prevent a pollinator-dependent plant species from expanding its geographic range (Liu et al. 2015), predictions of range shifts of plant species under climate change must consider not only the environmental requirements of plant species, but those of its pollinators as well. Climate change can lead to geographic and phenological mismatches between plants and their pollinators (Polce et al. 2014), so the long-term persistence of pollinator-dependent plants will require that their pollinators exhibit similar spatial and/or temporal shifts in response to changing climatic conditions. In all plant species, abiotic as well as biotic factors such as herbivory, parasitism and diseases may influence their realised geographic range. Among species pollinated by specialists, however, it is critical to consider the prospective range shift of their pollinators as well.

The current study has implications for assisted migration or *ex situ* conservation of threatened plants (Loss et al. 2011; Hällfors et al. 2016). Most *ex situ* conservation or assisted migration of threatened plants have focused only on the plants per se, with little or no concern for their pollinators. However, our results indicate that the introduction of effective pollinators to *ex situ* plant populations may be important for their long-term success. This is especially critical where habitats are fragmented, which increases isolation between suitable habitats. If pollinators cannot surmount barriers to dispersal, they will not spontaneously reach sites of *ex situ* conservation or assisted migration, resulting in reproductive failure in newly established plant populations. Furthermore, the abiotic environments of *ex situ* populations might not be favourable to the life cycle of the targeted pollinators. We suggest, if necessary, introducing effective substitute pollinators adapted to the abiotic environments of *ex situ* populations, provided that rigorous testing is conducted to ensure that there are no negative ecological consequences of such introduction. If no effective pollinators can be established sympatrically with an *ex situ* plant population, then hand-pollination will be necessary to induce sexual reproduction.

Conclusions

Biotic factors may limit plant species' distributions, and most established cases have identified limits due to interspecific competition and herbivory. The current study provides evidence that the absence of a single pollinator species can also limit a species' range. We found that *Iris ensata* is a partially self-compatible outcrosser, and in the southern portion of its geographic range, its sexual reproduction depends on the bumblebee *B. trifasciatus*. The current study detected marked

inbreeding depression for seed set and germination. Pollen limited seed set of individual flowers is common and was closely related to pollinator deficiency in the *ex situ* populations observed adjacent to the native range of *I. ensata*, suggesting that *B. trifasciatus* might mediate its seed production and thus the potential for regeneration. Pollinator limitation appears to prevent range expansion into sites where *ex situ* populations of *I. ensata* can grow well and flower normally. In summary, sexual reproductive failure caused by pollinator limitation is one factor that may restrict range expansion in suitable habitats in the studied region.

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Notes on contributors

Yue-E Xiao is interested in reproductive biology, population genetics and phylogeography of *Iris* spp. and orchids.

Dongmei Jin is a postdoctoral researcher interested in plant evolutionary ecology.

Kai Jiang is interested in population genetics and phylogeography of *Machilus* spp. and orchids. He completed part of the field work and data analyses.

Yong-Hong Hu is a senior scientist; he studies ecology and evolution of *Iris* spp. and *Paeonia* spp.

Xin Tong is a postdoctoral researcher interested in population ecology and molecular ecology.

Susan J. Mazer is interested in the mechanisms by which plants adapt to the ecological risks and opportunities that they encounter, and in the genetic constraints that may limit the rate or degree of adaptation.

Xiao-Yong Chen is interested in ecology and evolution of plants and their closely associated insects.

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